

ESTROGEN'S IMPACT ON COLON TUMOR FORMATION

A Senior Scholars Thesis

by

KIRBY TINSLEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Nutrition

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Approved by:

Research Advisor:
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Clinton Allred
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ABSTRACT

Estrogen's Impact on Colon Tumor Formation. (April 2010)

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One hundred thirty six men and women die every day from colon cancer in the United States (2008 statistics). Estrogen has been shown to reduce colonic tumor inflammation; however, it is unclear in the tumor development and growth process what is the most effective time for estrogen treatment. To gain a complete understanding of estrogen's chemopreventative effects, more research needs to be conducted. This study will demonstrate that when estrogen is given after colon tumors are fully initiated, the risk of the colon tumors continued growth is reduced. Tumors were induced in ovariectomized mice using azoxymethane (AOM). Ten weeks after tumors formed, an estrogen or control (VEH) pellet was inserted into the mice. Ten weeks later, the tumors and the most distal uninvolved tissues were removed. Apoptosis and proliferation assays were completed on all tissues. Results show that estrogen decreases colon tumor formation and area. Apoptosis and proliferation data will be presented. These data are important and have immediate clinical significance because estrogen therapy is most commonly used in post-menopausal women who are the highest risk of being diagnosed with colon cancer.

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CHAPTER I

INTRODUCTION

Colon cancer is the third most prevalent cancer in the United States, as well as ranking third among cancer-related causes of death (1). Colon cancer occurs in a higher percentage of men than women (2), possibly due to the presence of estrogen (E₂) in women as a protective effect.

Results from the Women's Health Initiative study showed that post-menopausal women on hormone replacement therapy (HRT) had a lower incidence of colon cancer than those receiving placebo treatment (3). A meta-analysis of eighteen epidemiologic studies showed a 20% decrease in colon tumor occurrence in postmenopausal women on HRT (4). A cohort study recently conducted found that a reduced amount of E₂ in menopausal cancer survivors was associated with an increased risk of a second colon tumor and they had a poorer prediction for recovery because these tumors were classified as grade 3 (one grade below the worst diagnosis) (5). A large amount of clinical studies show that HRT and estrogen replacement therapy (ERT) can reduce the risk of colon cancer in post-menopausal women, but there is no difference in effectiveness of these two therapies (6-11).

This thesis follows the style of Cancer Research.

The protective effects of E₂ are also shown in animal data. Smirnoff et al. found that rats injected with E₂ had fewer colon tumors than the control group (12). In another study, a metabolic precursor of E₂ was able to reduce colon tumor occurrence following exposure to a carcinogen (13). The ability of estrogen's protective effects during the progression stage of colon cancer has been researched (14), but the majority of data from other studies, as well as from Dr. Allred's lab (15 and 16), suggests that E₂ would cause a decrease in formation of pre-malignant lesions after carcinogen exposure in mice.

Constant inflammation in colonic epithelia is associated with an increased risk of tumor formation (17). This is shown by one of the most serious complications arising from inflammatory bowel disease (IBD) being colorectal cancer (18). One area that more information is needed on is the possible effect E₂ may have on tissue inflammation in the colon. The inflammation being the cause of the cancer is shown through different characteristics of tumors in IBD patients vs. tumors in non-IBD patients (19). Other studies have demonstrated that E₂ can reduce inflammation in cells from other tissues (20), but no studies have focused on the effect it might have on cells from colon tissues.

Recently, we completed an in vivo study in which E₂'s influence on the development of inflammation-associated colon tumor formation was examined. We found that E₂ reduced the number and size of colon tumors formed. We present the analysis of the tissues collected from those animals and seek to conclude how E₂ influences the cellular physiology of colon epithelial cells. We will concentrate specifically on how E₂

modifies cellular proliferation and programmed cell death (apoptosis), and this information will be presented.

CHAPTER II

MATERIALS AND METHODS

Animal study information

Mature female mice (C57BL/6) were ovariectomized before treatment so estrogen levels could be perfectly controlled. Tumors were induced by injecting 12.5 mg/kg AOM (azoxymethane). One week later, the first of two six-day treatments of 2.5% DSS (dextran sulphate sodium) in the mice's drinking water began. The two treatments were separated by three weeks. After 10 days of this treatment, an E₂ implant (1 mg E₂ and 19 mg cholesterol) or VEH pellet (20 mg cholesterol) was inserted into the back of the neck of the mice. The mice were killed ten weeks after the insertion of the pellet. Colons of the mice were cut out and the tumors and most distal uninvolved tissue were collected. The tissues were fixed in 4% PFA (paraformaldehyde) for four hours and then treated with ethanol overnight.

Apoptosis stain

Millipore ApopTag peroxidase in situ Apoptosis Detection Kit was used. Manufactures' instructions were followed with the following modifications: tissues were treated with 10 ug/mL proteinase K for 3 minutes at 37°C and endogenous peroxidase was blocked using .3% hydrogen peroxide in methanol for 30 minutes. DAB (diaminobenzidine) was used as chromogen and nuclei were stained in .5% methyl green for 5 seconds. Tissues were then cover slipped for analysis.

Proliferation stain

Endogenous peroxidase was quenched in .3% hydrogen peroxide in methanol for 30 minutes then citrate buffer. Tissues were placed in microwave on high for 10 minutes and medium for 10 minutes for antigen retrieval. The primary antibody was anti-brdu at 1:20 dilution overnight at 40°C in humidified chamber. The secondary antibody was goat anti-mouse HRP conjugated at 1:200 dilution for 1 hour at room temperature in humidified chamber. DAB was used as chromogen. Tissues were counterstained in Meyers Hematoxylin for 15 seconds. Tissues were then dehydrated and cover slipped for analysis.

Analysis

All tissues were analyzed the same after staining. Pictures of all tissues were taken, data was blinded, and a percentage was calculated. For tumor tissues, up to 250 cells in each the four different regions of the tumor was counted and a mean percentage of apoptotic/proliferative cells per tumor was calculated. For normal tissue, one side of the crypts was counted until 20 crypts were evaluated and a mean percentage of apoptotic/proliferative cells were calculated.

CHAPTER III

RESULTS

In the uninvolved tissue of the C57BL/6 mice, estrogen showed a 0.5% increase in the amount of apoptosis compared to the VEH mice (Fig. 1). The colon tumors that were removed were then analyzed to calculate the power of estrogen to promote apoptosis in formed tumors in comparison to the control mice. We found that the percentage of apoptotic cells was actually higher in the mice with the VEH pellet rather than the estrogen pellet (Fig. 2). A 0.5% difference for tumor apoptosis was calculated.

Proliferation in the uninvolved tissue increased by 3.5% in the estrogen treated mice (Fig. 3). A $p \leq 0.05$ was achieved in all of these results. The data shown here has mixed

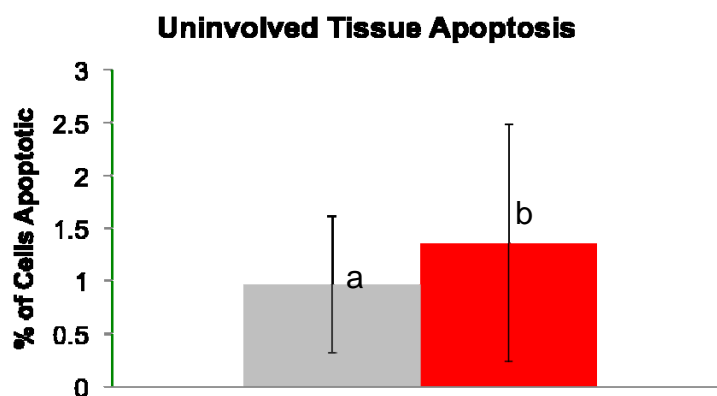


Figure 1. Apoptosis in the colon of C57BL/6 mice. Female mice were ovariectomized and tumors were induced by a 12.5mg/kg injection of AOM. One week later, mice were administered the first of two six day regiments of 2.5% dextran sulfate sodium (DSS) in the drinking water. Regiments were separated by three weeks. Ten days after the final DSS regiment, animals were implanted with either an E₂ (1mg E₂ + 19mg cholesterol) or VEH (20mg cholesterol) pellet. Animals were sacrificed 10 weeks after the final DSS treatment. Values are mean percentage of apoptotic cells per crypt +/- standard error. Twenty crypts were evaluated per animal: n=6 animals in the VEH group and n=7 animals in the E₂ group.

results of satisfaction to us. The increase of apoptotic cells in the uninvolved tissue was what we expected to see; however the two levels for estrogen treated tumors should have been more similar. The proliferation data from the uninvolved tissues is problematic. We are waiting for the analysis of proliferative cells in the tumors before any speculations are made as to why estrogen increased proliferation in uninvolved tissue.. Our studies demonstrated estrogen's protective effects by reducing the overall number of adenocarcinomas (Fig. 4) present in estrogen treated mice, as well as showing that estrogen reduced the overall mean area of the tumors (Fig. 5).

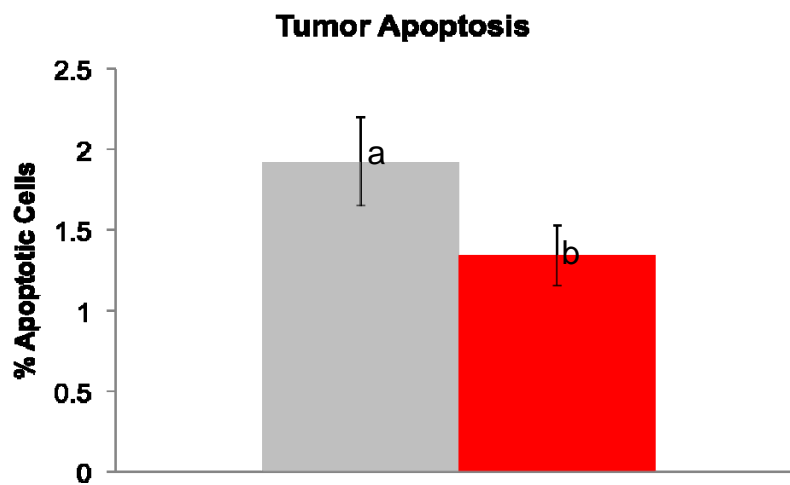


Figure 2. Apoptosis in the colon tumors of C57BL/6 mice. Female mice were ovariectomized and tumors were induced by a 12.5mg/kg injection of AOM. One week later, mice were administered the first of two six day regiments of 2.5% dextran sulfate sodium (DSS) in the drinking water. Regiments were separated by three weeks. Ten days after the final DSS regiment, animals were implanted with either an E₂ (1mg E₂ + 19mg cholesterol) or VEH (20mg cholesterol) pellet. Animals were sacrificed 10 weeks after the final DSS treatment. Values are mean percentage of apoptotic cells per tumor \pm standard error, 250 cells from four regions of each tumor were evaluated: n=48 tumors in the VEH group and n=50 tumors in the E₂ group. Bars without a common letter differ, $p \leq 0.05$.

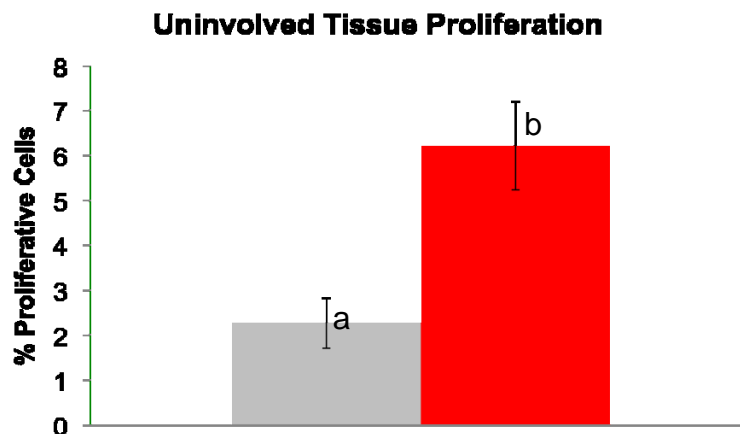


Figure 3. Proliferation in the colon of C57BL/6 mice. Female mice were ovariectomized and tumors were induced by a 12.5mg/kg injection of AOM. One week later, mice were administered the first of two six day regiments of 2.5% dextran sulfate sodium (DSS) in the drinking water. Regiments were separated by three weeks. Ten days after the final DSS regiment, animals were implanted with either an E₂ (1mg E₂ + 19mg cholesterol) or VEH (20mg cholesterol) pellet. Animals were sacrificed 10 weeks after the final DSS treatment. Values are mean percentage of proliferative cells per crypt +/- standard error. Twenty crypts were evaluated per animal: n=6 animals in the VEH group and n=7 animals in the E₂ group. Bars without a common letter differ, $p \leq 0.05$.

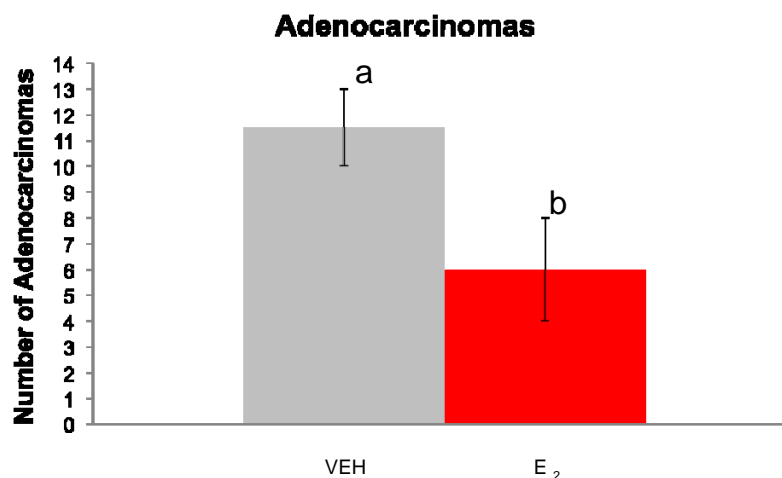


Figure 4. Tumors in the colon of C57BL/6 mice. Female mice were ovariectomized and tumors were induced by a 12.5mg/kg injection of AOM. One week later, mice were administered the first of two six day regiments of 2.5% dextran sulfate sodium (DSS) in the drinking water. Regiments were separated by three weeks. Ten days after the final DSS regiment, animals were implanted with either an E₂ (1mg E₂ + 19mg cholesterol) or VEH (20mg cholesterol) pellet. Animals were sacrificed 10 weeks after the final DSS treatment. Values are median number of tumors per animal +/- median absolute deviation, n=6 animals in the VEH group and n=7 animals in the E₂ group. Bars without a common letter differ, $p \leq 0.05$.

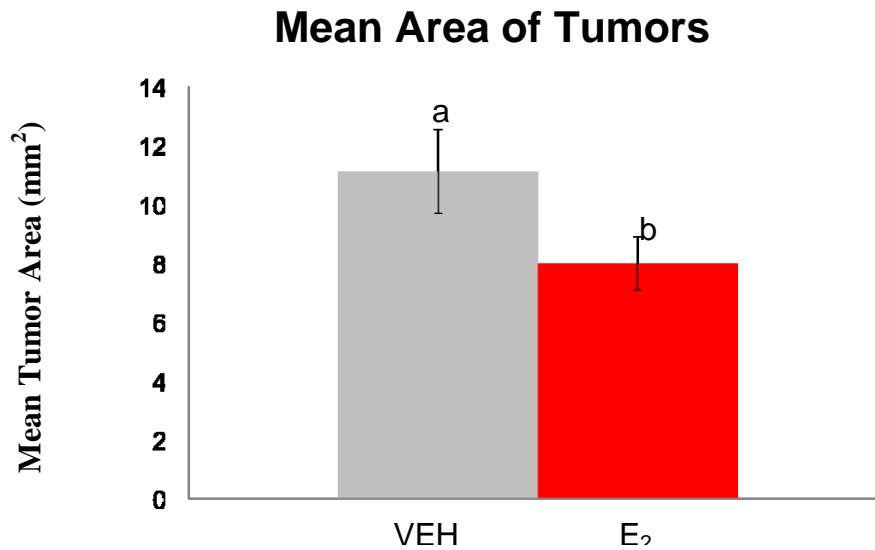


Figure 5. Tumors area in the colon of C57BL/6 mice. Female mice were ovariectomized and tumors were induced by a 12.5mg/kg injection of AOM. One week later, mice were administered the first of two six day regiments of 2.5% dextran sulfate sodium (DSS) in the drinking water. Regiments were separated by three weeks. Ten days after the final DSS regiment, animals were implanted with either an E₂ (1mg E₂ + 19mg cholesterol) or VEH (20mg cholesterol) pellet. Animals were sacrificed 10 weeks after the final DSS treatment. Values are mean area of tumors +/- standard error, n=34 tumors in the VEH group and n=29 tumors in the E₂ group. Bars without a common letter differ, $p \leq 0.05$.

CHAPTER IV

CONCLUSION

The mixed outcomes from other studies researching estrogen's effects on colon cancer formation has led to the need for more studies to be conducted, specifically on how estrogen affects non-malignant colon cellular physiology. From our studies thus far, it is easy to see that estrogen has an impact on colon tumor formation. We have shown that estrogen's presence in inflammation-associated colon tumors helps in the reduction of adenocarcinomas and mean area of tumors. After this positive outcome, we focused on proving that estrogen can alter physiology by inducing apoptosis and decreasing proliferation of the pre-cancerous cells. The apoptotic and proliferation data is mixed and thus may warrant more research. Estrogen changes the physiology of non-malignant cells to stop the transformation to malignant cells. The increase in percentage of apoptosis in the uninvolved tissue after estrogen treatment is what we wanted to see; however, the percentage difference in the tumor apoptosis is a little surprising. The tumor cell lines are non-responsive to estrogen treatment, so we expected to see the two levels stay the same after treating fully formed colon tumors with estrogen. Difference in this data and previous supports the idea that estrogen induces apoptosis in non-malignant colonocytes, but once the cells are cancerous estrogen no longer has an effect. We are waiting to calculate the percentage of proliferation in tumors before we try to come up with any final conclusion as to why estrogen treatment induced proliferation. We proved a definite protective effect of estrogen by showing its ability to reduce number and size

of colon tumors. That data has proved that estrogen is a chemopreventative agent. More research is needed to fully understand estrogen's effects on colon epithelial cells.

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